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IMMUNEX CORPORATION   2835-A

5   **TITLE**

                                  CYTOKINE THAT INDUCES APOPTOSIS

                                  CROSS-REFERENCE TO RELATED APPLICATIONS

10   This application is a continuation-in-part of application serial no. 08/496,632,
filed June 29, 1995, currently pending.

                                  BACKGROUND OF THE INVENTION
                The programmed cell death known as apoptosis is distinct from cell death due to
15   necrosis. Apoptosis occurs in embryogenesis, metamorphosis, endocrine-dependent
tissue atrophy, normal tissue turnover, and death of immune thymocytes (induced
through their antigen-receptor complex or by glucocorticoids) (Itoh et al., *Cell* 66:233,
1991). During maturation of T-cells in the thymus, T-cells that recognize self-antigens
are destroyed through the apoptotic process, whereas others are positively selected.
20   The possibility that some T-cells recognizing certain self epitopes (e.g., inefficiently
processed and presented antigenic determinants of a given self protein) escape this
elimination process and subsequently play a role in autoimmune diseases has been
suggested (Gammon et al., *Immunology Today* 12:193, 1991).
                A cell surface antigen known as Fas has been reported to mediate apoptosis and
25   is believed to play a role in clonal deletion of self-reactive T-cells (Itoh et al., *Cell*
66:233, 1991; Watanabe-Fukunage et al., *Nature* 356:314, 1992). Cross-linking a
specific monoclonal antibody to Fas has been reported to induce various cell lines to
undergo apoptosis (Yonehara et al., *J. Exp. Med.*, 169:1747, 1989; Trauth et al.,
Science, 245:301, 1989). However, under certain conditions, binding of a specific
30   monoclonal antibody to Fas can have a costimulatory effect on freshly isolated T cells
(Alderson et al., *J. Exp. Med.* 178:2231, 1993).
                DNAs encoding a rat Fas ligand (Suda et al., *Cell*, 75:1169, 1993) and a human
Fas ligand (Takahashi et al., *International Immunology* 6:1567, 1994) have been
isolated. Binding of the Fas ligand to cells expressing Fas antigen has been
35   demonstrated to induce apoptosis (Suda et al., *supra*, and Takahashi et al., *supra*).
                Investigation into the existence and identity of other molecule(s) that play a role
in apoptosis is desirable. Identifying such molecules would provide an additional

means of regulating apoptosis, as well as providing further insight into the development of self-tolerance by the immune system and the etiology of autoimmune diseases.

SUMMARY OF THE INVENTION

5   The present invention provides a novel cytokine protein, as well as isolated DNA encoding the cytokine and expression vectors comprising the isolated DNA. Properties of the novel cytokine, which is a member of the tumor necrosis factor (TNF) family of ligands, include the ability to induce apoptosis of certain types of target cells. This protein thus is designated TNF Related Apoptosis Inducing Ligand (TRAIL).

10   Among the types of cells that are killed by contact with TRAIL are cancer cells such as leukemia, lymphoma, and melanoma cells, and cells infected with a virus.

A method for producing TRAIL polypeptides involves culturing host cells transformed with a recombinant expression vector that contains TRAIL-encoding DNA under conditions appropriate for expression of TRAIL, then recovering the expressed

15   TRAIL polypeptide from the culture. Antibodies directed against TRAIL polypeptides are also provided.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 presents the results of an assay described in example 8. The assay

20   demonstrated that a soluble human TRAIL polypeptide induced death of Jurkat cells, which are a leukemia cell line.

Figure 2 presents the results of an assay described in example 11. Contact with a soluble human TRAIL polypeptide induced death of cytomegalovirus-infected human fibroblasts, whereas non-virally infected fibroblasts were not killed.

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DETAILED DESCRIPTION OF THE INVENTION

A novel protein designated TRAIL is provided herein, along with DNA encoding TRAIL and recombinant expression vectors comprising TRAIL DNA. A method for producing recombinant TRAIL polypeptides involves cultivating host cells

30   transformed with the recombinant expression vectors under conditions appropriate for expression of TRAIL, and recovering the expressed TRAIL.

The present invention also provides antibodies that specifically bind TRAIL proteins. In one embodiment, the antibodies are monoclonal antibodies.

The TRAIL protein induces apoptosis of certain types of target cells, such as

35   transformed cells that include but are not limited to cancer cells and virally-infected cells. As demonstrated in examples 5, 8, 9, and 10 below, TRAIL induced apoptosis of human leukemia, lymphoma, and melanoma cell lines. Among the uses of TRAIL is

use in killing cancer cells. TRAIL finds further use in treatment of viral infections. Infection with cytomegalovirus (CMV) rendered human fibroblasts susceptible to apoptosis when contacted with TRAIL, whereas uninfected fibroblasts were not killed through contact with TRAIL (see example 11).

5   Isolation of a DNA encoding human TRAIL is described in example 1 below. The nucleotide sequence of the human TRAIL DNA isolated in example 1 is presented in SEQ ID NO:1, and the amino acid sequence encoded thereby is presented in SEQ ID NO:2. This human TRAIL protein comprises an N-terminal cytoplasmic domain (amino acids 1-18), a transmembrane region (amino acids 19-38), and an extracellular

10   domain (amino acids 39-281). The extracellular domain contains a receptor-binding region.

E. coli strain DH10B cells transformed with a recombinant vector containing this human TRAIL DNA were deposited with the American Type Culture Collection on June 14, 1995, and assigned accession no. 69849. The deposit was made under the

15   terms of the Budapest Treaty. The recombinant vector in the deposited strain is the expression vector pDC409 (described in example 5). The vector was digested with SalI and NotI, and human TRAIL DNA that includes the entire coding region shown in SEQ ID NO:1 was ligated into the vector.

DNA encoding a second human TRAIL protein was isolated as described in

20   example 2. The nucleotide sequence of this DNA is presented in SEQ ID NO:3, and the amino acid sequence encoded thereby is presented in SEQ ID NO:4. The encoded protein comprises an N-terminal cytoplasmic domain (amino acids 1-18), a transmembrane region (amino acids 19-38), and an extracellular domain (amino acids 39-101).

25   The DNA of SEQ ID NO:3 lacks a portion of the DNA of SEQ ID NO:1, and is thus designated the human TRAIL deletion variant (huTRAILdv) clone. Nucleotides 18 through 358 of SEQ ID NO:1 are identical to nucleotides 8 through 348 of the huTRAILdv DNA of SEQ ID NO:3. Nucleotides 359 through 506 of SEQ ID NO:1 are missing from the cloned DNA of SEQ ID NO:3. The deletion causes a shift in the

30   reading frame, which results in an in-frame stop codon after amino acid 101 of SEQ ID NO:4. The DNA of SEQ ID NO:3 thus encodes a truncated protein. Amino acids 1 through 90 of SEQ ID NO:2 are identical to amino acids 1 through 90 of SEQ ID NO:4. However, due to the deletion, the C-terminal portion of the huTRAILdv protein (amino acids 91 through 101 of SEQ ID NO:4) differs from the residues in the corresponding positions in SEQ ID NO:2. In contrast to the full length huTRAIL protein, the

35   truncated huTRAILdv protein does not exhibit the ability to induce apoptosis of the T cell leukemia cells of the Jurkat cell line.

DNA encoding a mouse TRAIL protein has also been isolated, as described in example 3. The nucleotide sequence of this DNA is presented in SEQ ID NO:5 and the amino acid sequence encoded thereby is presented in SEQ ID NO:6. The encoded protein comprises an N-terminal cytoplasmic domain (amino acids 1-17), a transmembrane region (amino acids 18-38), and an extracellular domain (amino acids 39-291). This mouse TRAIL is 64% identical to the human TRAIL of SEQ ID NO:2 at the amino acid level. The coding region of the mouse TRAIL nucleotide sequence is 75% identical to the coding region of the human nucleotide sequence of SEQ ID NO:1.

One embodiment of the present invention is directed to human TRAIL protein characterized by the N-terminal amino acid sequence MetAlaMetMetGluValGlnGly GlyProSerLeuGlyGlnThr (amino acids 1-15 of SEQ ID NOS:2 and 4). Mouse TRAIL proteins characterized by the N-terminal amino acid sequence MetProSerSerGlyAla LeuLysAspLeuSerPheSerGlnHis (amino acids 1-15 of SEQ ID NO:6) are also provided herein.

The TRAIL of the present invention is distinct from the protein known as Fas ligand (Suda et al., *Cell*, 75:1169, 1993; Takahashi et al., *International Immunology* 6:1567, 1994). Fas ligand induces apoptosis of certain cell types, *via* the receptor known as Fas. As demonstrated in example 5, TRAIL-induced apoptosis of target cells is not mediated through Fas. The human TRAIL amino acid sequence of SEQ ID NO:2 is about 20% identical to the human Fas ligand amino acid sequence that is presented in Takahashi et al., *supra*. The extracellular domain of human TRAIL is about 28.4% identical to the extracellular domain of human Fas ligand.

The amino acid sequences disclosed herein reveal that TRAIL is a member of the TNF family of ligands (Smith et al. *Cell*, 73:1349, 1993; Suda et al., *Cell*, 75:1169, 1993; Smith et al., *Cell*, 76:959, 1994). The percent identities between the human TRAIL extracellular domain amino acid sequence and the amino acid sequence of the extracellular domain of other proteins of this family are as follows: 28.4% with Fas ligand, 22.4% with lymphotoxin-β, 22.9% with TNF-α, 23.1% with TNF-β, 22.1% with CD30 ligand, and 23.4% with CD40 ligand.

TRAIL was tested for ability to bind receptors of the TNF-R family of receptors. The binding analysis was conducted using the slide autoradiography procedure of Gearing et al. (*EMBO J.* 8:3667, 1989). The analysis revealed no detectable binding of human TRAIL to human CD30, CD40, 4-1BB, OX40, TNF-R (p80 form), CD27, or LTβR (also known as TNFR-RP). The results in example 5 indicate that human TRAIL does not bind human Fas.

The TRAIL polypeptides of the present invention include polypeptides having amino acid sequences that differ from, but are highly homologous to, those presented

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in SEQ ID NOS:2 and 6. Examples include, but are not limited to, homologs derived from other mammalian species, variants (both naturally occurring variants and those generated by recombinant DNA technology), and TRAIL fragments that retain a desired biological activity. Such polypeptides exhibit a biological activity of the TRAIL

5   proteins of SEQ ID NOS:2 and 6, and preferably comprise an amino acid sequence that is at least 80% identical (most preferably at least 90% identical) to the amino acid sequence presented in SEQ ID NO:2 or SEQ ID NO:6. These embodiments of the present invention are described in more detail below.

Conserved sequences located in the C-terminal portion of proteins in the TNF

10   family are identified in Smith et al. (*Cell*, 73:1349, 1993, see page 1353 and Figure 6); Suda et al. (*Cell*, 75:1169, 1993, see figure 7); Smith et al. (*Cell*, 76:959, 1994, see figure 3); and Goodwin et al. (*Eur. J. Immunol.*, 23:2631, 1993, see figure 7 and pages 2638-39), hereby incorporated by reference. Among the amino acids in the human TRAIL protein that are conserved (in at least a majority of TNF family

15   members) are those in positions 124-125 (AH), 136 (L), 154 (W), 169 (L), 174 (L), 180 (G), 182 (Y), 187 (Q), 190 (F), 193 (Q), and 275-276 (FG) of SEQ ID NO:2. Another structural feature of TRAIL is a spacer region between the C-terminus of the trans-membrane region and the portion of the extracellular domain that is believed to be most important for biological activity. This spacer region, located at the N-terminus of

20   the extracellular domain, consists of amino acids 39 through 94 of SEQ ID NO:2. Analogous spacers are found in other family members, e.g., CD40 ligand. Amino acids 138 through 153 correspond to a loop between the ß sheets of the folded (three dimensional) human TRAIL protein.

Provided herein are membrane-bound TRAIL proteins (comprising a

25   cytoplasmic domain, a transmembrane region, and an extracellular domain) as well as TRAIL fragments that retain a desired biological property of the full length TRAIL protein. In one embodiment, TRAIL fragments are soluble TRAIL polypeptides comprising all or part of the extracellular domain, but lacking the transmembrane region that would cause retention of the polypeptide on a cell membrane. Soluble TRAIL

30   proteins are capable of being secreted from the cells in which they are expressed. Advantageously, a heterologous signal peptide is fused to the N-terminus such that the soluble TRAIL is secreted upon expression.

Soluble TRAIL may be identified (and distinguished from its non-soluble membrane-bound counterparts) by separating intact cells which express the desired

35   protein from the culture medium, e.g., by centrifugation, and assaying the medium (supernatant) for the presence of the desired protein. The presence of TRAIL in the medium indicates that the protein was secreted from the cells and thus is a soluble form

of the TRAIL protein. Naturally-occurring soluble forms of TRAIL are encompassed by the present invention.

The use of soluble forms of TRAIL is advantageous for certain applications. Purification of the proteins from recombinant host cells is facilitated, since the soluble proteins are secreted from the cells. Further, soluble proteins are generally more suitable for intravenous administration.

Examples of soluble TRAIL polypeptides are those containing the entire extracellular domain (e.g., amino acids 39 to 281 of SEQ ID NO:2 or amino acids 39 to 291 of SEQ ID NO:6). Fragments of the extracellular domain that retain a desired biological activity are also provided. Such fragments advantageously include regions of TRAIL that are conserved in proteins of the TNF family of ligands, as described above.

Additional examples of soluble TRAIL polypeptides are those lacking not only the cytoplasmic domain and transmembrane region, but also all or part of the above-described spacer region. Soluble human TRAIL polypeptides thus include, but are not limited to, polypeptides comprising amino acids x to 281, wherein x represents any of the amino acids in positions 39 through 95 of SEQ ID NO:2. In the embodiment in which residue 95 is the N-terminal amino acid, the entire spacer region has been deleted.

TRAIL fragments, including soluble polypeptides, may be prepared by any of a number of conventional techniques. A DNA sequence encoding a desired TRAIL fragment may be subcloned into an expression vector for production of the TRAIL fragment. The TRAIL-encoding DNA sequence advantageously is fused to a sequence encoding a suitable leader or signal peptide. The desired TRAIL-encoding DNA fragment may be chemically synthesized using known techniques. DNA fragments also may be produced by restriction endonuclease digestion of a full length cloned DNA sequence, and isolated by electrophoresis on agarose gels. If necessary, oligonucleotides that reconstruct the 5' or 3' terminus to a desired point may be ligated to a DNA fragment generated by restriction enzyme digestion. Such oligonucleotides may additionally contain a restriction endonuclease cleavage site upstream of the desired coding sequence, and position an initiation codon (ATG) at the N-terminus of the coding sequence.

The well known polymerase chain reaction (PCR) procedure also may be employed to isolate and amplify a DNA sequence encoding a desired protein fragment. Oligonucleotides that define the desired termini of the DNA fragment are employed as 5' and 3' primers. The oligonucleotides may additionally contain recognition sites for restriction endonucleases, to faciliate insertion of the amplified DNA fragment into an

expression vector. PCR techniques are described in Saiki et al., *Science* 239:487 (1988); *Recombinant DNA Methodology*, Wu et al., eds., Academic Press, Inc., San Diego (1989), pp. 189-196; and *PCR Protocols: A Guide to Methods and Applications*, Innis et al., eds., Academic Press, Inc. (1990).

5   As will be understood by the skilled artisan, the transmembrane region of each TRAIL protein discussed above is identified in accordance with conventional criteria for identifying that type of hydrophobic domain. The exact boundaries of a transmembrane region may vary slightly (most likely by no more than five amino acids on either end) from those presented above. Computer programs useful for identifying such

10   hydrophobic regions in proteins are available.

      The TRAIL DNA of the present invention includes cDNA, chemically synthesized DNA, DNA isolated by PCR, genomic DNA, and combinations thereof. Genomic TRAIL DNA may be isolated by hybridization to the TRAIL cDNA disclosed herein using standard techniques. RNA transcribed from the TRAIL DNA is also

15   encompassed by the present invention.

      A search of the NCBI databank identified five expressed sequence tags (ESTs) having regions of identity with TRAIL DNA. These ESTs (NCBI accession numbers T90422, T82085, T10524, R31020, and Z36726) are all human cDNA fragments. The NCBI records do not disclose any polypeptide encoded by the ESTs, and do not

20   indicate what the reading frame, if any, might be. However, even if the knowledge of the reading frame revealed herein by disclosure of complete TRAIL coding regions is used to express the ESTs, none of the encoded polypeptides would have the apoptosis-inducing property of the presently-claimed TRAIL polypeptides. In other words, if each of the five ESTs were inserted into expression vectors downstream from an

25   initiator methionine codon, in the reading frame elucidated herein, none of the resulting expressed polypeptides would contain a sufficient portion of the extracellular domain of TRAIL to induce apoptosis of Jurkat cells.

      Certain embodiments of the present invention provide isolated DNA comprising a nucleotide sequence selected from the group consisting of nucleotides 88 to 933 of

30   SEQ ID NO:1 (human TRAIL coding region); nucleotides 202 to 933 of SEQ ID NO:1 (encoding the human TRAIL extracellular domain); nucleotides 47 to 922 of SEQ ID NO:5 (mouse TRAIL coding region); and nucleotides 261 to 922 of SEQ ID NO:5 (encoding the mouse TRAIL extracellular domain). DNAs encoding biologically active fragments of the proteins of SEQ ID NOS:2 and 6 are also provided. Further

35   embodiments include sequences comprising nucleotides 370 to 930 of SEQ ID NO:1 and nucleotides 341 to 919 of SEQ ID NO:5, which encode the particular human and murine soluble TRAIL polypeptides, respectively, described in example 7.

Due to degeneracy of the genetic code, two DNA sequences may differ, yet encode the same amino acid sequence. The present invention thus provides isolated DNA sequences encoding biologically active TRAIL, selected from DNA comprising the coding region of a native human or murine TRAIL cDNA, or fragments thereof,

5   and DNA which is degenerate as a result of the genetic code to the native TRAIL DNA sequence.

Also provided herein are purified TRAIL polypeptides, both recombinant and non-recombinant. Variants and derivatives of native TRAIL proteins that retain a desired biological activity are also within the scope of the present invention. In one

10   embodiment, the biological activity of an TRAIL variant is essentially equivalent to the biological activity of a native TRAIL protein. One desired biological activity of TRAIL is the ability to induce death of Jurkat cells. Assay procedures for detecting apoptosis of target cells are well known. DNA laddering is among the characteristics of cell death via apoptosis, and is recognized as one of the observable phenomena that distinguish

15   apoptotic cell death from necrotic cell death. Examples of assay techniques suitable for detecting death or apoptosis of target cells include those described in examples 5 and 8 to 11. Another property of TRAIL is the ability to bind to Jurkat cells.

TRAIL variants may be obtained by mutations of native TRAIL nucleotide sequences, for example. A TRAIL variant, as referred to herein, is a polypeptide

20   substantially homologous to a native TRAIL, but which has an amino acid sequence different from that of native TRAIL because of one or a plurality of deletions, insertions or substitutions. TRAIL-encoding DNA sequences of the present invention encompass sequences that comprise one or more additions, deletions, or substitutions of nucleotides when compared to a native TRAIL DNA sequence, but that encode an

25   TRAIL protein that is essentially biologically equivalent to a native TRAIL protein.

The variant amino acid or DNA sequence preferably is at least 80% identical to a native TRAIL sequence, most preferably at least 90% identical. The degree of homology (percent identity) between a native and a mutant sequence may be determined, for example, by comparing the two sequences using computer programs

30   commonly employed for this purpose. One suitable program is the GAP computer program, version 6.0, described by Devereux et al. (*Nucl. Acids Res.* 12:387, 1984) and available from the University of Wisconsin Genetics Computer Group (UWGCG). The GAP program utilizes the alignment method of Needleman and Wunsch (*J. Mol. Biol.* 48:443, 1970), as revised by Smith and Waterman (*Adv. Appl. Math* 2:482,

35   1981). Briefly, the GAP program defines identity as the number of aligned symbols (i.e., nucleotides or amino acids) which are identical, divided by the total number of symbols in the shorter of the two sequences. The preferred default parameters for the

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GAP program include: (1) a unary comparison matrix (containing a value of 1 for identities and 0 for non-identities) for nucleotides, and the weighted comparison matrix of Gribskov and Burgess, *Nucl. Acids Res.* 14:6745, 1986, as described by Schwartz and Dayhoff, eds., *Atlas of Protein Sequence and Structure*, National Biomedical Research Foundation, pp. 353-358, 1979; (2) a penalty of 3.0 for each gap and an additional 0.10 penalty for each symbol in each gap; and (3) no penalty for end gaps.

Alterations of the native amino acid sequence may be accomplished by any of a number of known techniques. Mutations can be introduced at particular loci by synthesizing oligonucleotides containing a mutant sequence, flanked by restriction sites enabling ligation to fragments of the native sequence. Following ligation, the resulting reconstructed sequence encodes an analog having the desired amino acid insertion, substitution, or deletion.

Alternatively, oligonucleotide-directed site-specific mutagenesis procedures can be employed to provide an altered gene having particular codons altered according to the substitution, deletion, or insertion required. Techniques for making such alterations include those disclosed by Walder et al. (*Gene* 42:133, 1986); Bauer et al. (*Gene* 37:73, 1985); Craik (*BioTechniques*, January 1985, 12-19); Smith et al. (*Genetic Engineering: Principles and Methods*, Plenum Press, 1981); and U.S. Patent Nos. 4,518,584 and 4,737,462, which are incorporated by reference herein.

Variants may comprise conservatively substituted sequences, meaning that one or more amino acid residues of a native TRAIL polypeptide are replaced by different residues, but that the conservatively substituted TRAIL polypeptide retains a desired biological activity that is essentially equivalent to that of a native TRAIL polypeptide. Examples of conservative substitutions include substitution of amino acids that do not alter the secondary and/or tertiary structure of TRAIL. Other examples involve substitution of amino acids outside of the receptor-binding domain, when the desired biological activity is the ability to bind to a receptor on target cells and induce apoptosis of the target cells. A given amino acid may be replaced by a residue having similar physiochemical characteristics, e.g., substituting one aliphatic residue for another (such as Ile, Val, Leu, or Ala for one another), or substitution of one polar residue for another (such as between Lys and Arg; Glu and Asp; or Gln and Asn). Other such conservative substitutions, e.g., substitutions of entire regions having similar hydrophobicity characteristics, are well known. TRAIL polypeptides comprising conservative amino acid substitutions may be tested in one of the assays described herein to confirm that a desired biological activity of a native TRAIL is retained. DNA sequences encoding TRAIL polypeptides that contain such conservative amino acid substitutions are encompassed by the present invention.

Conserved amino acids located in the C-terminal portion of proteins in the TNF family, and believed to be important for biological activity, have been identified. These conserved sequences are discussed in Smith et al. (*Cell*, 73:1349, 1993, see page 1353 and Figure 6); Suda et al. (*Cell*, 75:1169, 1993, see figure 7); Smith et al. (*Cell*, 76:959, 1994, see figure 3); and Goodwin et al. (*Eur. J. Immunol.*, 23:2631, 1993, see figure 7 and pages 2638-39). Advantageously, the conserved amino acids are not altered when generating conservatively substituted sequences. If altered, amino acids found at equivalent positions in other members of the TNF family are substituted.

TRAIL also may be modified to create TRAIL derivatives by forming covalent or aggregative conjugates with other chemical moieties, such as glycosyl groups, lipids, phosphate, acetyl groups and the like. Covalent derivatives of TRAIL may be prepared by linking the chemical moieties to functional groups on TRAIL amino acid side chains or at the N-terminus or C-terminus of a TRAIL polypeptide or the extracellular domain thereof. Other derivatives of TRAIL within the scope of this invention include covalent or aggregative conjugates of TRAIL or its fragments with other proteins or polypeptides, such as by synthesis in recombinant culture as N-terminal or C-terminal fusions. For example, the conjugate may comprise a signal or leader polypeptide sequence (e.g. the α-factor leader of *Saccharomyces*) at the N-terminus of a TRAIL polypeptide. The signal or leader peptide co-translationally or post-translationally directs transfer of the conjugate from its site of synthesis to a site inside or outside of the cell membrane or cell wall.

TRAIL polypeptide fusions can comprise peptides added to facilitate purification and identification of TRAIL. Such peptides include, for example, poly-His or the antigenic identification peptides described in U.S. Patent No. 5,011,912 and in Hopp et al., *Bio/Technology* 6:1204, 1988. One such peptide is the FLAG® peptide, Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys (DYKDDDDK) (SEQ ID NO:7), which is highly antigenic and provides an epitope reversibly bound by a specific monoclonal antibody, thus enabling rapid assay and facile purification of expressed recombinant protein. This sequence is also specifically cleaved by bovine mucosal enterokinase at the residue immediately following the Asp-Lys pairing. Fusion proteins capped with this peptide may also be resistant to intracellular degradation in *E. coli*.

A murine hybridoma designated 4E11 produces a monoclonal antibody that binds the peptide DYKDDDDK (SEQ ID NO:7) in the presence of certain divalent metal cations (as described in U.S. Patent 5,011,912), and has been deposited with the American Type Culture Collection under accession no HB 9259. Expression systems useful for producing recombinant proteins fused to the FLAG® peptide, as well as monoclonal antibodies that bind the peptide and are useful in purifying the recombinant

proteins, are available from Eastman Kodak Company, Scientific Imaging Systems, New Haven, Connecticut.

The present invention further includes TRAIL polypeptides with or without associated native-pattern glycosylation. TRAIL expressed in yeast or mammalian

5   expression systems may be similar to or significantly different from a native TRAIL polypeptide in molecular weight and glycosylation pattern, depending upon the choice of expression system. Expression of TRAIL polypeptides in bacterial expression systems, such as *E. coli*, provides non-glycosylated molecules.

Glycosylation sites in the TRAIL extracellular domain can be modified to

10 preclude glycosylation while allowing expression of a homogeneous, reduced carbohydrate analog using yeast or mammalian expression systems. N-glycosylation sites in eukaryotic polypeptides are characterized by an amino acid triplet Asn-X-Y, wherein X is any amino acid except Pro and Y is Ser or Thr. Appropriate modifications to the nucleotide sequence encoding this triplet will result in

15 substitutions, additions or deletions that prevent attachment of carbohydrate residues at the Asn side chain. Known procedures for inactivating N-glycosylation sites in proteins include those described in U.S. Patent 5,071,972 and EP 276,846. A potential N-glycosylation site is found at positions 109-111 in the human protein of SEQ ID NO:2 and at positions 52-54 in the murine protein of SEQ ID NO:6.

20   In another example, sequences encoding Cys residues that are not essential for biological activity can be altered to cause the Cys residues to be deleted or replaced with other amino acids, preventing formation of incorrect intramolecular disulfide bridges upon renaturation. Other variants are prepared by modification of adjacent dibasic amino acid residues to enhance expression in yeast systems in which KEX2 protease

25 activity is present. EP 212,914 discloses the use of site-specific mutagenesis to inactivate KEX2 protease processing sites in a protein. KEX2 protease processing sites are inactivated by deleting, adding or substituting residues to alter Arg-Arg, Arg-Lys, and Lys-Arg pairs to eliminate the occurrence of these adjacent basic residues. Lys-Lys pairings are considerably less susceptible to KEX2 cleavage, and

30 conversion of Arg-Lys or Lys-Arg to Lys-Lys represents a conservative and preferred approach to inactivating KEX2 sites. Potential KEX2 protease processing sites are found at positions 89-90 and 149-150 in the protein of SEQ ID NO:2, and at positions 85-86, 135-136, and 162-163 in the protein of SEQ ID NO:6.

Naturally occurring TRAIL variants are also encompassed by the present

35 invention. Examples of such variants are proteins that result from alternative mRNA splicing events (since TRAIL is encoded by a multi-exon gene) or from proteolytic cleavage of the TRAIL protein, wherein a desired biological activity is retained.

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Alternative splicing of mRNA may yield a truncated but biologically active TRAIL protein, such as a naturally occurring soluble form of the protein, for example. Variations attributable to proteolysis include, for example, differences in the N- or C-termini upon expression in different types of host cells, due to proteolytic removal of one or more terminal amino acids from the TRAIL protein. In addition, proteolytic cleavage may release a soluble form of TRAIL from a membrane-bound form of the protein. Allelic variants are also encompassed by the present invention.

Oligomers

The present invention encompasses TRAIL polypeptides in the form of oligomers, such as dimers, trimers, or higher oligomers. Oligomers may be formed by disulfide bonds between cysteine residues on different TRAIL polypeptides, or by non-covalent interactions between TRAIL polypeptide chains, for example. In other embodiments, oligomers comprise from two to four TRAIL polypeptides joined *via* covalent or non-covalent interactions between peptide moieties fused to the TRAIL polypeptides. Such peptides may be peptide linkers (spacers), or peptides that have the property of promoting oligomerization. Leucine zippers and certain polypeptides derived from antibodies are among the peptides that can promote oligomerization of TRAIL polypeptides attached thereto, as described in more detail below. The TRAIL polypeptides preferably are soluble.

Preparation of fusion proteins comprising heterologous polypeptides fused to various portions of antibody-derived polypeptides (including the Fc domain) has been described, e.g., by Ashkenazi et al. (*PNAS USA* 88:10535, 1991); Byrn et al. (*Nature* 344:667, 1990); and Hollenbaugh and Aruffo ("Construction of Immunoglobulin Fusion Proteins", in *Current Protocols in Immunology, Supplement 4*, pages 10.19.1 - 10.19.11, 1992), hereby incorporated by reference. In one embodiment of the invention, an TRAIL dimer is created by fusing TRAIL to an Fc region polypeptide derived from an antibody. The term "Fc polypeptide" includes native and mutein forms, as well as truncated Fc polypeptides containing the hinge region that promotes dimerization. The Fc polypeptide preferably is fused to a soluble TRAIL (e.g., comprising only the extracellular domain).

A gene fusion encoding the TRAIL/Fc fusion protein is inserted into an appropriate expression vector. The TRAIL/Fc fusion proteins are allowed to assemble much like antibody molecules, whereupon interchain disulfide bonds form between the Fc polypeptides, yielding divalent TRAIL. In other embodiments, TRAIL may be substituted for the variable portion of an antibody heavy or light chain. If fusion

proteins are made with both heavy and light chains of an antibody, it is possible to form an TRAIL oligomer with as many as four TRAIL extracellular regions.

One suitable Fc polypeptide is the native Fc region polypeptide derived from a human IgG1, which is described in PCT application WO 93/10151, hereby incorporated by reference. Another useful Fc polypeptide is the Fc mutein described in U.S. Patent application serial no. 08/097,827. The amino acid sequence of the mutein is identical to that of the native Fc sequence presented in WO 93/10151, except that amino acid 19 has been changed from Leu to Ala, amino acid 20 has been changed from Leu to Glu, and amino acid 22 has been changed from Gly to Ala. This mutein Fc exhibits reduced affinity for immunoglobulin receptors.

Alternatively, oligomeric TRAIL may comprise two or more soluble TRAIL polypeptides joined through peptide linkers. Examples include those peptide linkers described in United States Patent 5,073,627 (hereby incorporated by reference). Fusion proteins comprising multiple TRAIL polypeptides separated by peptide linkers may be produced using conventional recombinant DNA technology.

Another method for preparing oligomeric TRAIL polypeptides involves use of a leucine zipper. Leucine zipper domains are peptides that promote oligomerization of the proteins in which they are found. Leucine zippers were originally identified in several DNA-binding proteins (Landschulz et al., *Sicence* 240:1759, 1988), and have since been found in a variety of different proteins. Among the known leucine zippers are naturally occurring peptides and derivatives thereof that dimerize or trimerize. Examples of leucine zipper domains suitable for producing soluble oligomeric TRAIL proteins are those described PCT application WO 94/10308, hereby incorporated by reference. Recombinant fusion proteins comprising a soluble TRAIL polypeptide fused to a peptide that dimerizes or trimerizes in solution are expressed in suitable host cells, and the resulting soluble oligomeric TRAIL is recovered from the culture supernatant.

Certain members of the TNF family of proteins are believed to exist in trimeric form (Beutler and Huffel, *Science* 264:667, 1994; Banner et al., *Cell* 73:431, 1993). Thus, trimeric TRAIL may offer the advantage of enhanced biological activity. Preferred leucine zipper moieties are those that preferentially form trimers. One example is a leucine zipper derived from lung surfactant protein D (SPD), as described in Hoppe et al. (*FEBS Letters* 344:191, 1994) and in U.S. Patent application serial no. 08/446,922, hereby incorporated by reference. Other peptides derived from naturally occurring trimeric proteins may be employed in preparing trimeric TRAIL.

As described in example 7, a soluble Flag®-TRAIL polypeptide expressed in CV-1/EBNA cells spontaneously formed oligomers believed to be a mixture of dimers and trimers. The cytotoxic effect of this soluble Flag®-TRAIL in the assay of example

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8 was enhanced by including an anti-Flag® antibody, possibly because the antibody facilitated cross-linking of TRAIL/receptor complexes. In one embodiment of the invention, biological activity of TRAIL is enhanced by employing TRAIL in conjunction with an antibody that is capable of cross-linking TRAIL. Cells that are to

5   be killed may be contacted with both a soluble TRAIL polypeptide and such an antibody.

As one example, cancer or virally infected cells are contacted with an anti-Flag® antibody and a soluble Flag®-TRAIL polypeptide. Preferably, an antibody fragment lacking the Fc region is employed. Bivalent forms of the antibody may bind the Flag®

10   moieties of two soluble Flag®-TRAIL polypeptides that are found in separate dimers or trimers. The antibody may be mixed or incubated with a Flag®-TRAIL polypeptide prior to administration *in vivo*.

Expression Systems

15   The present invention provides recombinant expression vectors for expression of TRAIL, and host cells transformed with the expression vectors. Any suitable expression system may be employed. The vectors include a DNA encoding a TRAIL polypeptide, operably linked to suitable transcriptional or translational regulatory nucleotide sequences, such as those derived from a mammalian, microbial, viral, or

20   insect gene. Examples of regulatory sequences include transcriptional promoters, operators, or enhancers, an mRNA ribosomal binding site, and appropriate sequences which control transcription and translation initiation and termination. Nucleotide sequences are operably linked when the regulatory sequence functionally relates to the TRAIL DNA sequence. Thus, a promoter nucleotide sequence is operably linked to an

25   TRAIL DNA sequence if the promoter nucleotide sequence controls the transcription of the TRAIL DNA sequence. An origin of replication that confers the ability to replicate in the desired host cells, and a selection gene by which transformants are identified, are generally incorporated into the expression vector.

In addition, a sequence encoding an appropriate signal peptide can be

30   incorporated into expression vectors. A DNA sequence for a signal peptide (secretory leader) may be fused in frame to the TRAIL sequence so that the TRAIL is initially translated as a fusion protein comprising the signal peptide. A signal peptide that is functional in the intended host cells promotes extracellular secretion of the TRAIL polypeptide. The signal peptide is cleaved from the TRAIL polypeptide upon secretion

35   of TRAIL from the cell.

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Suitable host cells for expression of TRAIL polypeptides include prokaryotes, yeast or higher eukaryotic cells. Appropriate cloning and expression vectors for use with bacterial, fungal, yeast, and mammalian cellular hosts are described, for example, in Pouwels et al. *Cloning Vectors: A Laboratory Manual,* Elsevier, New York,

5   (1985). Cell-free translation systems could also be employed to produce TRAIL polypeptides using RNAs derived from DNA constructs disclosed herein.

Prokaryotes include gram negative or gram positive organisms, for example, *E. coli* or *Bacilli*. Suitable prokaryotic host cells for transformation include, for example, *E. coli, Bacillus subtilis, Salmonella typhimurium,* and various other species within the

10   genera *Pseudomonas, Streptomyces,* and *Staphylococcus.* In a prokaryotic host cell, such as *E. coli,* a TRAIL polypeptide may include an N-terminal methionine residue to facilitate expression of the recombinant polypeptide in the prokaryotic host cell. The N-terminal Met may be cleaved from the expressed recombinant TRAIL polypeptide.

Expression vectors for use in prokaryotic host cells generally comprise one or

15   more phenotypic selectable marker genes. A phenotypic selectable marker gene is, for example, a gene encoding a protein that confers antibiotic resistance or that supplies an autotrophic requirement. Examples of useful expression vectors for prokaryotic host cells include those derived from commercially available plasmids such as the cloning vector pBR322 (ATCC 37017). pBR322 contains genes for ampicillin and tetracycline

20   resistance and thus provides simple means for identifying transformed cells. An appropriate promoter and a TRAIL DNA sequence are inserted into the pBR322 vector. Other commercially available vectors include, for example, pKK223-3 (Pharmacia Fine Chemicals, Uppsala, Sweden) and pGEM1 (Promega Biotec, Madison, WI, USA).

Promoter sequences commonly used for recombinant prokaryotic host cell

25   expression vectors include β-lactamase (penicillinase), lactose promoter system (Chang et al., *Nature 275*:615, 1978; and Goeddel et al., *Nature 281*:544, 1979), tryptophan (trp) promoter system (Goeddel et al., *Nucl. Acids Res.* 8:4057, 1980; and EP-A-36776) and tac promoter (Maniatis, *Molecular Cloning: A Laboratory Manual,* Cold Spring Harbor Laboratory, p. 412, 1982). A particularly useful prokaryotic host cell

30   expression system employs a phage λ P_L promoter and a cI857ts thermolabile repressor sequence. Plasmid vectors available from the American Type Culture Collection which incorporate derivatives of the λ P_L promoter include plasmid pHUB2 (resident in *E. coli* strain JMB9, ATCC 37092) and pPLc28 (resident in *E. coli* RR1, ATCC 53082).

TRAIL alternatively may be expressed in yeast host cells, preferably from the

35   *Saccharomyces* genus (e.g., *S. cerevisiae*). Other genera of yeast, such as *Pichia* or *Kluyveromyces,* may also be employed. Yeast vectors will often contain an origin of replication sequence from a 2μ yeast plasmid, an autonomously replicating sequence

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(ARS), a promoter region, sequences for polyadenylation, sequences for transcription termination, and a selectable marker gene. Suitable promoter sequences for yeast vectors include, among others, promoters for metallothionein, 3-phosphoglycerate kinase (Hitzeman et al., *J. Biol. Chem. 255*:2073, 1980) or other glycolytic enzymes

5   (Hess et al., *J. Adv. Enzyme Reg. 7*:149, 1968; and Holland et al., *Biochem. 17*:4900, 1978), such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phospho-glucose isomerase, and glucokinase. Other suitable vectors and promoters for use in

10   yeast expression are further described in Hitzeman, EPA-73,657. Another alternative is the glucose-repressible ADH2 promoter described by Russell et al. (*J. Biol. Chem. 258*:2674, 1982) and Beier et al. (*Nature 300*:724, 1982). Shuttle vectors replicable in both yeast and *E. coli* may be constructed by inserting DNA sequences from pBR322 for selection and replication in *E. coli* (Amp^r gene and origin of replication) into the

15   above-described yeast vectors.

The yeast α-factor leader sequence may be employed to direct secretion of the TRAIL polypeptide. The α-factor leader sequence is often inserted between the promoter sequence and the structural gene sequence. *See,* e.g., Kurjan et al., *Cell 30*:933, 1982 and Bitter et al., *Proc. Natl. Acad. Sci. USA 81*:5330, 1984. Other

20   leader sequences suitable for facilitating secretion of recombinant polypeptides from yeast hosts are known to those of skill in the art. A leader sequence may be modified near its 3' end to contain one or more restriction sites. This will facilitate fusion of the leader sequence to the structural gene.

Yeast transformation protocols are known to those of skill in the art. One such

25   protocol is described by Hinnen et al., *Proc. Natl. Acad. Sci. USA 75*:1929, 1978. The Hinnen et al. protocol selects for Trp$^+$ transformants in a selective medium, wherein the selective medium consists of 0.67% yeast nitrogen base, 0.5% casamino acids, 2% glucose, 10 μg/ml adenine and 20 μg/ml uracil.

Yeast host cells transformed by vectors containing an ADH2 promoter sequence

30   may be grown for inducing expression in a "rich" medium. An example of a rich medium is one consisting of 1% yeast extract, 2% peptone, and 1% glucose supplemented with 80 μg/ml adenine and 80 μg/ml uracil. Derepression of the ADH2 promoter occurs when glucose is exhausted from the medium.

Mammalian or insect host cell culture systems could also be employed to

35   express recombinant TRAIL polypeptides. Bacculovirus systems for production of heterologous proteins in insect cells are reviewed by Luckow and Summers, *Bio/Technology* 6:47 (1988). Established cell lines of mammalian origin also may be

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employed. Examples of suitable mammalian host cell lines include the COS-7 line of monkey kidney cells (ATCC CRL 1651) (Gluzman et al., *Cell 23*:175, 1981), L cells, C127 cells, 3T3 cells (ATCC CCL 163), Chinese hamster ovary (CHO) cells, HeLa cells, and BHK (ATCC CRL 10) cell lines, and the CVI/EBNA cell line derived from

5 the African green monkey kidney cell line CVI (ATCC CCL 70) as described by McMahan et al. (*EMBO J.* 10: 2821, 1991).

Transcriptional and translational control sequences for mammalian host cell expression vectors may be excised from viral genomes. Commonly used promoter sequences and enhancer sequences are derived from Polyoma virus, Adenovirus 2,

10 Simian Virus 40 (SV40), and human cytomegalovirus. DNA sequences derived from the SV40 viral genome, for example, SV40 origin, early and late promoter, enhancer, splice, and polyadenylation sites may be used to provide other genetic elements for expression of a structural gene sequence in a mammalian host cell. Viral early and late promoters are particularly useful because both are easily obtained from a viral genome

15 as a fragment which may also contain a viral origin of replication (Fiers et al., *Nature 273*:113, 1978). Smaller or larger SV40 fragments may also be used, provided the approximately 250 bp sequence extending from the *Hind* III site toward the *Bgl* I site located in the SV40 viral origin of replication site is included.

Expression vectors for use in mammalian host cells can be constructed as

20 disclosed by Okayama and Berg (*Mol. Cell. Biol. 3*:280, 1983), for example. A useful system for stable high level expression of mammalian cDNAs in C127 murine mammary epithelial cells can be constructed substantially as described by Cosman et al. (*Mol. Immunol.* 23:935, 1986). A high expression vector, PMLSV N1/N4, described by Cosman et al., *Nature 312*:768, 1984 has been deposited as ATCC 39890.

25 Additional examples of mammalian expression vectors are described in EP-A-0367566, and in WO 91/18982. The vectors may be derived from retroviruses.

For expression of TRAIL, a type II protein lacking a native signal sequence, a heterologous signal sequence or leader functional in mammalian host cells may be added. Examples include the signal sequence for interleukin-7 (IL-7) described in

30 United States Patent 4,965,195, the signal sequence for interleukin-2 receptor described in Cosman et al., *Nature* 312:768 (1984); the interleukin-4 receptor signal peptide described in EP 367,566; the type I interleukin-1 receptor signal peptide described in U.S. Patent 4,968,607; and the type II interleukin-1 receptor signal peptide described in EP 460,846.

35 A preferred expression system employs a leader sequence derived from cytomegalovirus. Example 7 illustrates the use of one such leader. In example 7, mammalian host cells were transformed with an expression vector encoding the peptide

Met Ala Arg Arg Leu Trp Ile Leu Ser Leu Leu Ala Val Thr Leu Thr Val Ala Leu Ala Ala Pro Ser Gln Lys Ser Lys Arg Arg Thr Ser Ser (SEQ ID NO:9) fused to the N-terminus of an octapeptide designated FLAG® (SEQ ID NO:7, described above), which in turn is fused to the N-terminus of a soluble TRAIL polypeptide. Residues 1 through 29 of

5   SEQ ID NO:9 constitute a CMV-derived leader sequence, whereas residues 30 through 32 are encoded by oligonucleotides employed in constructing the expression vector described in example 7.

Expression systems that employ such CMV-derived leader peptides are useful for expressing proteins other than TRAIL. Expression vectors comprising a DNA

10   sequence that encodes amino acids 1 through 29 of SEQ ID NO:9 are provided herein. In another embodiment, the vector comprises a sequence that encodes amino acids 1 through 28 of SEQ ID NO:9. DNA encoding a desired heterologous protein is positioned downstream of, and in the same reading frame as, DNA encoding the leader. Additional residues (e.g., those encoded by linkers or primers) may be encoded by

15   DNA positioned between the sequences encoding the leader and the desired heterologous protein, as illustrated by the vector described in example 7. As is understood in the pertinent field, the expression vectors comprise promoters and any other desired regulatory sequences, operably linked to the sequences encoding the leader and heterologous protein.

20   The leader peptide presented in SEQ ID NO:9 is cleaved after the arginine residue at position 29 to yield the mature secreted form of a protein fused thereto. Alternatively or additionally, cleavage may occur between amino acids 20 and 21, or between amino acids 28 and 29, of SEQ ID NO:9.

A method for producing a heterologous recombinant protein involves culturing

25   mammalian host cells transformed with such an expression vector under conditions that promote expression and secretion of the heterologous protein, and recovering the protein from the culture medium. Expression systems employing CMV leaders may be used to produce any desired protein, examples of which include, but are not limited to, colony stimulating factors, interferons, interleukins, other cytokines, and cytokine

30   receptors.

Purified TRAIL Protein

The present invention provides purified TRAIL proteins, which may be produced by recombinant expression systems as described above or purified from

35   naturally occurring cells. The desired degree of purity may depend on the intended use of the protein. A relatively high degree of purity is desired when the protein is to be administered *in vivo*, for example. Advantageously, TRAIL polypeptides are purified

such that no protein bands corresponding to other proteins are detectable by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). It will be recognized by one skilled in the pertinent field that multiple bands corresponding to TRAIL protein may be detected by SDS-PAGE, due to differential glycosylation, variations in post-

5   translational processing, and the like, as discussed above. A preparation of TRAIL protein is considered to be purified as long as no bands corresponding to different (non-TRAIL) proteins are visualized. TRAIL most preferably is purified to substantial homogeneity, as indicated by a single protein band upon analysis by SDS-PAGE. The protein band may be visualized by silver staining, Coomassie blue staining, or (if the

10   protein is radiolabeled) by autoradiography.

One process for producing the TRAIL protein comprises culturing a host cell transformed with an expression vector comprising a DNA sequence that encodes TRAIL under conditions such that TRAIL is expressed. The TRAIL protein is then recovered from the culture (from the culture medium or cell extracts). As the skilled

15   artisan will recognize, procedures for purifying the recombinant TRAIL will vary according to such factors as the type of host cells employed and whether or not the TRAIL is secreted into the culture medium.

For example, when expression systems that secrete the recombinant protein are employed, the culture medium first may be concentrated using a commercially available

20   protein concentration filter, for example, an Amicon or Millipore Pellicon ultrafiltration unit. Following the concentration step, the concentrate can be applied to a purification matrix such as a gel filtration medium. Alternatively, an anion exchange resin can be employed, for example, a matrix or substrate having pendant diethylaminoethyl (DEAE) groups. The matrices can be acrylamide, agarose, dextran, cellulose or other

25   types commonly employed in protein purification. Alternatively, a cation exchange step can be employed. Suitable cation exchangers include various insoluble matrices comprising sulfopropyl or carboxymethyl groups. Sulfopropyl groups are preferred. Finally, one or more reversed-phase high performance liquid chromatography (RP-HPLC) steps employing hydrophobic RP-HPLC media, (e.g., silica gel having

30   pendant methyl or other aliphatic groups) can be employed to further purify TRAIL. Some or all of the foregoing purification steps, in various combinations, can be employed to provide a purified TRAIL protein.

Recombinant protein produced in bacterial culture may be isolated by initial disruption of the host cells, centrifugation, extraction from cell pellets if an insoluble

35   polypeptide, or from the supernatant fluid if a soluble polypeptide, followed by one or more concentration, salting-out, ion exchange, affinity purification or size exclusion chromatography steps. Finally, RP-HPLC can be employed for final purification

steps. Microbial cells can be disrupted by any convenient method, including freeze-thaw cycling, sonication, mechanical disruption, or use of cell lysing agents.

Transformed yeast host cells are preferably employed to express TRAIL as a secreted polypeptide. This simplifies purification. Secreted recombinant polypeptide
5   from a yeast host cell fermentation can be purified by methods analogous to those disclosed by Urdal et al. (*J. Chromatog.* *296*:171, 1984). Urdal et al. describe two sequential, reversed-phase HPLC steps for purification of recombinant human IL-2 on a preparative HPLC column.

Alternatively, TRAIL polypeptides can be purified by immunoaffinity
10   chromatography. An affinity column containing an antibody that binds TRAIL may be prepared by conventional procedures and employed in purifying TRAIL. Example 4 describes a procedure for generating monoclonal antibodies directed against TRAIL.

Properties and Uses of TRAIL

15   Programmed cell death (apoptosis) occurs during embryogenesis, metamorphosis, endocrine-dependent tissue atrophy, normal tissue turnover, and death of immune thymocytes. Regulation of programmed cell death is vital for normal functioning of the immune system. To illustrate, T cells that recognize self-antigens are destroyed through the apoptotic process during maturation of T-cells in the thymus,
20   whereas other T cells are positively selected. The possibility that some T-cells recognizing certain self epitopes (e.g., inefficiently processed and presented antigenic determinants of a given self protein) escape this elimination process and subsequently play a role in autoimmune diseases has been proposed (Gammon et al., *Immunology Today* 12:193, 1991).

25   Insufficient apoptosis has been implicated in certain conditions, while elevated levels of apoptotic cell death have been associated with other diseases. The desirability of identifying and using agents that regulate apoptosis in treating such disorders is recognized (Kromer, *Advances in Immunology*, 58:211, 1995; Groux et al., *J. Exp. Med.* 175:331, 1992; Sachs and Lotem, *Blood* 82:15, 1993).

30   Abnormal resistance of T cells toward undergoing apoptosis has been linked to lymphocytosis, lymphadenopathy, splenomegaly, accumulation of self-reactive T cells, autoimmune disease, development of leukemia, and development of lymphoma (Kromer, *supra*; see especially pages 214-215). Conversely, excessive apoptosis of T cells has been suggested to play a role in lymphopenia, systemic immunodeficiency,
35   and specific immunodeficiency, with specific examples being virus-induced immunodeficient states associated with infectious mononucleosis and cytomegalovirus infection, and tumor-mediated immunosuppression (Kromer, *supra*; see especially page

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214). Depletion of CD4$^+$ T cells in HIV-infected individuals may be attributable to inappropriate activation-induced cell death (AICD) by apoptosis (Groux et al., *J. Exp. Med.* 175:331, 1992).

As demonstrated in examples 5 and 8, TRAIL induces apoptosis of the acute T cell leukemia cell line designated Jurkat clone E6-1. TRAIL thus is a research reagent useful in studies of apoptosis, including the regulation of programmed cell death. Since Jurkat cells are a leukemia cell line arising from T cells, the TRAIL of the present invention finds use in studies of the role TRAIL may play in apoptosis of other transformed T cells, such as other malignant cell types arising from T cells.

TRAIL binds Jurkat cells, as well as inducing apoptosis thereof. TRAIL did not cause death of freshly isolated murine thymocytes, or peripheral blood T cells (PBTs) freshly extracted from a healthy human donor. A number of uses flow from these properties of TRAIL.

TRAIL polypeptides may be used to purify leukemia cells, or any other cell type to which TRAIL binds. Leukemia cells may be isolated from a patient's blood, for example. In one embodiment, the cells are purified by affinity chromatography, using a chromatography matrix having TRAIL bound thereto. The TRAIL attached to the chromatography matrix may be a full length protein, an TRAIL fragment comprising the extracellular domain, an TRAIL-containing fusion protein, or other suitable TRAIL polypeptide described herein. In one embodiment, a soluble TRAIL/Fc fusion protein is bound to a Protein A or Protein G column through interaction of the Fc moiety with the Protein A or Protein G. Alternatively, TRAIL may be used in isolating leukemia cells by flow cytometry.

The thus-purified leukemia cells are expected to die following binding of TRAIL, but the dead cells will still bear cell surface antigens, and may be employed as immunogens in deriving anti-leukemia antibodies. The leukemia cells, or a desired cell surface antigen isolated therefrom, find further use in vaccine development.

Since TRAIL binds and kills leukemia cells (the Jurkat cell line), TRAIL also may be useful in treating leukemia. A therapeutic method involves contacting leukemia cells with an effective amount of TRAIL. In one embodiment, a leukemia patient's blood is contacted *ex vivo* with an TRAIL polypeptide. The TRAIL may be immobilized on a suitable matrix. TRAIL binds the leukemia cells, thus removing them from the patient's blood before the blood is returned into the patient.

Alternatively or additionally, bone marrow extracted from a leukemia patient may be contacted with an amount of TRAIL effective in inducing death of leukemia cells in the bone marrow. Bone marrow may be aspirated from the sternum or iliac

crests, for example, and contacted with TRAIL to purge leukemia cells. The thus-treated marrow is returned to the patient.

TRAIL also binds to, and induces apoptosis of, lymphoma and melanoma cells (see examples 5, 9, and 10). Thus, uses of TRAIL that are analogous to those described above for leukemia cells are applicable to lymphoma and melanoma cells. TRAIL polypeptides may be employed in treating cancer, including, but not limited to, leukemia, lymphoma, and melanoma. In one embodiment, the lymphoma is Burkitt's lymphoma. Table I in example 9 shows that TRAIL had a cytotoxic effect on several Burkitt's lymphoma cell lines. Epstein-Barr virus is an etiologic agent of Burkitt's lymphoma.

TRAIL polypeptides also find use in treating viral infections. Contact with TRAIL caused death of cells infected with cytomegalovirus, but not of the same cell type when uninfected, as described in example 11. The ability of TRAIL to kill cells infected with other viruses can be confirmed using the assay described in example 11. Such viruses include, but are not limited to, encephalomyocarditis virus, Newcastle disease virus, vesicular stomatitis virus, herpes simplex virus, adenovirus-2, bovine viral diarrhea virus, HIV, and Epstein-Barr virus.

An effective amount of TRAIL is administered to a mammal, including a human, afflicted with a viral infection. In one embodiment, TRAIL is employed in conjunction with interferon to treat a viral infection. In the experiment described in example 11, pretreatment of CMV-infected cells with γ-interferon enhanced the level of killing of the infected cells that was mediated by TRAIL. TRAIL may be administered in conjunction with other agents that exert a cytotoxic effect on cancer cells or virus-infected cells.

In another embodiment, TRAIL is used to kill virally infected cells in cell preparations, tissues, or organs that are to be transplanted. To illustrate, bone marrow may be contacted with TRAIL to kill virus infected cells that may be present therein, before the bone marrow is transplanted into the recipient.

The TRAIL of the present invention may be used in developing treatments for any disorder mediated (directly or indirectly) by defective or insufficient amounts of TRAIL. A therapeutically effective amount of purified TRAIL protein is administered to a patient afflicted with such a disorder. Alternatively, TRAIL DNA sequences may be employed in developing a gene therapy approach to treating such disorders. Disclosure herein of native TRAIL nucleotide sequences permits the detection of defective TRAIL genes, and the replacement thereof with normal TRAIL-encoding genes. Defective genes may be detected in *in vitro* diagnostic assays, and by

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comparision of the native TRAIL nucleotide sequence disclosed herein with that of an TRAIL gene derived from a person suspected of harboring a defect in this gene.

The present invention provides pharmaceutical compositions comprising purified TRAIL and a physiologically acceptable carrier, diluent, or excipient. Suitable carriers, diluents, and excipients are nontoxic to recipients at the dosages and concentrations employed. Such compositions may comprise buffers, antioxidants such as ascorbic acid, low molecular weight (less than about 10 residues) polypeptides, proteins, amino acids, carbohydrates including glucose, sucrose or dextrins, chelating agents such as EDTA, glutathione and other stabilizers and excipients commonly employed in pharmaceutical compositions. Neutral buffered saline or saline mixed with conspecific serum albumin are among the appropriate diluents. The composition may be formulated as a lyophilizate using appropriate excipient solutions (e.g. sucrose) as diluents.

For therapeutic use, purified proteins of the present invention are administered to a patient, preferably a human, for treatment in a manner appropriate to the indication. Thus, for example, the pharmaceutical compositions can be administered locally, by intravenous injection, continuous infusion, sustained release from implants, or other suitable technique. Appropriate dosages and the frequency of administration will depend, of course, on such factors as the nature and severity of the indication being treated, the desired response, the condition of the patient and so forth.

The TRAIL protein employed in the pharmaceutical compositions preferably is purified such that the TRAIL protein is substantially free of other proteins of natural or endogenous origin, desirably containing less than about 1% by mass of protein contaminants residual of production processes. Such compositions, however, can contain other proteins added as stabilizers, carriers, excipients or co-therapeutics.

The TRAIL-encoding DNAs disclosed herein find use in the production of TRAIL polypeptides, as discussed above. Fragments of the TRAIL nucleotide sequences are also useful. In one embodiment, such fragments comprise at least about 17 consecutive nucleotides, more preferably at least 30 consecutive nucleotides, of the human or murine TRAIL DNA disclosed herein. DNA and RNA complements of said fragments are provided herein, along with both single-stranded and double-stranded forms of the TRAIL DNA of SEQ ID NOS:1, 3 and 5.

Among the uses of such TRAIL nucleic acid fragments are use as a probe or as primers in a polymerase chain reaction (PCR). As one example, a probe corresponding to the extracellular domain of TRAIL may be employed. The probes find use in detecting the presence of TRAIL nucleic acids in *in vitro* assays and in such procedures as Northern and Southern blots. Cell types expressing TRAIL can be identified as

well. Such procedures are well known, and the skilled artisan can choose a probe of suitable length, depending on the particular intended application. For PCR, 5' and 3' primers corresponding to the termini of a desired TRAIL DNA sequence are employed to isolate and amplify that sequence, using conventional techniques.

5   Other useful fragments of the TRAIL nucleic acids are antisense or sense oligonucleotides comprising a single-stranded nucleic acid sequence (either RNA or DNA) capable of binding to target TRAIL mRNA (sense) or TRAIL DNA (antisense) sequences. Such a fragment generally comprises at least about 14 nucleotides, preferably from about 14 to about 30 nucleotides. The ability to create an antisense or a

10   sense oligonucleotide, based upon a cDNA sequence for a given protein is described in, for example, Stein and Cohen, *Cancer Res.* 48:2659, 1988 and van der Krol et al., *BioTechniques* 6:958, 1988.

Binding of antisense or sense oligonucleotides to target nucleic acid sequences results in the formation of duplexes that block translation (RNA) or transcription

15   (DNA) by one of several means, including enhanced degradation of the duplexes, premature termination of transcription or translation, or by other means. The antisense oligonucleotides thus may be used to block expression of TRAIL proteins.

Antisense or sense oligonucleotides further comprise oligonucleotides having modified sugar-phosphodiester backbones (or other sugar linkages, such as those

20   described in WO91/06629) and wherein such sugar linkages are resistant to endogenous nucleases. Such oligonucleotides with resistant sugar linkages are stable *in vivo* (i.e., capable of resisting enzymatic degradation) but retain sequence specificity to be able to bind to target nucleotide sequences. Other examples of sense or antisense oligonucleotides include those oligonucleotides which are covalently linked to organic

25   moieties, such as those described in WO 90/10448, and other moieties that increases affinity of the oligonucleotide for a target nucleic acid sequence, such as poly-(L-lysine). Further still, intercalating agents, such as ellipticine, and alkylating agents or metal complexes may be attached to sense or antisense oligonucleotides to modify binding specificities of the antisense or sense oliginucleotide for the target nucleotide

30   sequence.

Antisense or sense oligonucleotides may be introduced into a cell containing the target nucleic acid sequence by any gene transfer method, including, for example, $CaPO_4$-mediated DNA transfection, electroporation, or other gene transfer vectors such as Epstein-Barr virus. Antisense or sense oligonucleotides are preferably introduced

35   into a cell containing the target nucleic acid sequence by insertion of the antisense or sense oligonucleotide into a suitable retroviral vector, then contacting the cell with the retrovirus vector containing the inserted sequence, either *in vivo* or *ex vivo*. Suitable

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retroviral vectors include, but are not limited to, the murine retrovirus M-MuLV, N2 (a retrovirus derived from M-MuLV), or or the double copy vectors designated DCT5A, DCT5B and DCT5C (see PCT Application US 90/02656). Alternatively, other promotor sequences may be used to express the oligonucleotide.

5   Sense or antisense oligonucleotides may also be introduced into a cell containing the target nucleotide sequence by formation of a conjugate with a ligand binding molecule, as described in WO 91/04753. Suitable ligand binding molecules include, but are not limited to, cell surface receptors, growth factors, other cytokines, or other ligands that bind to cell surface receptors. Preferably, conjugation of the
10   ligand binding molecule does not substantially interfere with the ability of the ligand binding molecule to bind to its corresponding molecule or receptor, or block entry of the sense or antisense oligonucleotide or its conjugated version into the cell.

         Alternatively, a sense or an antisense oligonucleotide may be introduced into a cell containing the target nucleic acid sequence by formation of an oligonucleotide-lipid
15   complex, as described in WO 90/10448. The sense or antisense oligonucleotide-lipid complex is preferably dissociated within the cell by an endogenous lipase.

Antibodies Immunoreactive with TRAIL

         The TRAIL proteins of the present invention, or immunogenic fragments thereof, may be employed in generating antibodies. The present invention thus
20   provides antibodies that specifically bind TRAIL, i.e., the antibodies bind to TRAIL *via* the antigen-binding sites of the antibody (as opposed to non-specific binding).

         Polyclonal and monoclonal antibodies may be prepared by conventional techniques. See, for example, *Monoclonal Antibodies, Hybridomas: A New*
25   *Dimension in Biological Analyses*, Kennet et al. (eds.), Plenum Press, New York (1980); and *Antibodies: A Laboratory Manual* , Harlow and Land (eds.), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, (1988). Production of monoclonal antibodies that are immunoreactive with TRAIL is further illustrated in example 4 below.

30   Antigen-binding fragments of such antibodies, which may be produced by conventional techniques, are also encompassed by the present invention. Examples of such fragments include, but are not limited to, Fab, F(ab'), and F(ab')$_2$ fragments. Antibody fragments and derivatives produced by genetic engineering techniques are also provided.

35   The monoclonal antibodies of the present invention include chimeric antibodies, e.g., humanized versions of murine monoclonal antibodies. Such humanized antibodies may be prepared by known techniques, and offer the advantage of reduced

immunogenicity when the antibodies are administered to humans. In one embodiment, a humanized monoclonal antibody comprises the variable region of a murine antibody (or just the antigen binding site thereof) and a constant region derived from a human antibody. Alternatively, a humanized antibody fragment may comprise the antigen binding site of a murine monoclonal antibody and a variable region fragment (lacking the antigen-binding site) derived from a human antibody. Procedures for the production of chimeric and further engineered monoclonal antibodies include those described in Riechmann et al. (*Nature 332*:323, 1988), Liu et al. (*PNAS 84*:3439, 1987), Larrick et al. (*Bio/Technology 7*:934, 1989), and Winter and Harris (*TIPS 14*:139, May, 1993).

Among the uses of the antibodies is use in assays to detect the presence of TRAIL polypeptides, either *in vitro* or *in vivo*. The antibodies find further use in purifying TRAIL by affinity chromatography. Those antibodies that additionally can block binding of TRAIL to target cells may be used to inhibit a biological activity of TRAIL. A therapeutic method involves *in vivo* administration of an amount of such an antibody that is effective in inhibiting a TRAIL-mediated biological activity. Disorders mediated or exacerbated by TRAIL, directly or indirectly, are thus treated.

Pharmaceutical compositions comprising an antibody that is immunoreactive with TRAIL, and a suitable, diluent, excipient, or carrier, are provided herein. Suitable components of such compositions are as described above for the compositions containing TRAIL proteins.

The following examples are provided to illustrate particular embodiments of the present invention, and are not to be construed as limiting the scope of the invention.

EXAMPLE 1: Isolation of a Human TRAIL DNA

DNA encoding a human TRAIL protein of the present invention was isolated by the following procedure. A TBLASTN search of the dbEST data base at the National Center for Biological Information (NCBI) was performed, using the query sequence LVVXXXGLYYVYXQVXF (SEQ ID NO:8). This sequence is based upon the most conserved region of the TNF ligand family (Smith et al., *Cell*, 73:1349, 1993). An expressed sequence tag (EST) file, GenBank accession number Z36726, was identified using these search parameters. The GenBank file indicated that this EST was obtained from a human heart atrium cDNA library.

Two 30-bp oligonucleotides based upon sequences from the 3' and 5' ends of this EST file were synthesized. The oligonucleotide from the 3' end had the sequence TGAAATCGAAAGTATGTTTGGGAATAGATG (complement of nucleotides 636 to 665 of SEQ ID NO:1) and the 5' oligonucleotide was TGACGAAGAGAGTATGAA

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CAGCCCCTGCTG (nucleotides 291 to 320 of SEQ ID NO:1). The oligonucleotides were 5' end labeled with ^{32}P γ-ATP and polynucleotide kinase. Two λgt10 cDNA libraries were screened by conventional methods with an equimolar mixture of these labeled oligonucleotides as probe. One library was a human heart 5' stretch cDNA

5   library (Stratagene Cloning Systems, La Jolla, CA). The other was a peripheral blood lymphocyte (PBL) library prepared as follows: PBLs were obtained from normal human volunteers and treated with 10 ng/ml of OKT3 (an anti-CD3 antibody) and 10 ng/ml of human IL-2 for six days. The PBL cells were washed and stimulated with 500 ng/ml of ionomycin (Calbiochem) and 10 ng/ml PMA for 4 hours. Messenger

10   RNA was isolated from the stimulated PBL cells. cDNA synthesized on the mRNA template was packaged into λgt10 phage vectors (Gigapak®, Stratagene Cloning Systems, La Jolla, CA).

Recombinant phages were plated onto *E. coli* strain C600-HFL and screened using standard plaque hybridization techniques. Nitrocellulose filters were lifted from

15   these plates in duplicate, and hybridized with the ^{32}P-labeled oligonucleotides overnight at 67°C in a solution of 60 mM Tris pH 8.0, 2 mM EDTA, 5x Denhardt's Solution, 6x SSC, 1 mg/ml n-lauroyl sarcosine, 0.5% NP40, and 4 µg/ml SS salmon sperm DNA. The filters were then washed in 3x SSC at 67°C for thirty minutes.

From the heart 5' stretch cDNA library, one positive plaque was obtained out of

20   approximately one million plaques. This clone did not include the 3' end of the gene. Using the PBL library, approximately 50 positive plaques were obtained out of 500,000 plaques. Fifteen of these first round positive plaques were picked, and the inserts from the enriched pools were amplified using oligonucleotide primers designed to amplify phage inserts. The resulting products were resolved by 1.5% agarose gel

25   electrophoresis, blotted onto nitrocellulose, and analyzed by standard Southern blot technique using the ^{32}P-labeled 30-mer oligonucleotides as probes. The two plaque picks that produced the largest bands by Southern analysis were purified by secondary screening, and isolated phage plaques were obtained using the same procedures described above.

30   DNA from the isolated phages was prepared by the plate lysis method, and the cDNA inserts were excised with EcoRI, purified by electrophoresis using 1.5% agarose in Tris-Borate-EDTA buffer, and ligated into the pBluescript® SK(+) plasmid. These inserts were then sequenced by conventional methods, and the resulting sequences were aligned.

35   The nucleotide sequence of a human TRAIL DNA is presented in SEQ ID NO:1 and the amino acid sequence encoded thereby is presented in SEQ ID NO:2. This human TRAIL protein comprises an N-terminal cytoplasmic domain (amino acids 1-

18), a transmembrane region (amino acids 19-38), and an extracellular domain (amino acids 39-281). The calculated molecular weight of this protein is 32,508 daltons.

E. coli strain DH10B cells transformed with a recombinant vector containing this TRAIL DNA were deposited with the American Type Culture Collection on June 14, 1995, and assigned accession no. 69849. The deposit was made under the terms of the Budapest Treaty. The recombinant vector in the deposited strain is the expression vector pDC409 (described in example 5). The vector was digested with SalI and NotI, and human TRAIL DNA that includes the entire coding region shown in SEQ ID NO:1 was ligated into the digested vector.

EXAMPLE 2: Isolation of DNA Encoding a Truncated TRAIL

DNA encoding a second human TRAIL protein was isolated as follows. This truncated TRAIL does not exhibit the ability to induce apoptosis of Jurkat cells.

PCR analysis, using the 30-mers described in example 1 as the 5' and 3' primers, indicated that 3 out of 14 of the first round plaque picks in example 1 contained shorter forms of the TRAIL DNA. One of the shortened forms of the gene was isolated, ligated into the pBluescript® SK(+) cloning vector (Stratagene Cloning Systems, La Jolla, CA) and sequenced.

The nucleotide sequence of this DNA is presented in SEQ ID NO:3. The amino acid sequence encoded thereby is presented in SEQ ID NO:4. The encoded protein comprises an N-terminal cytoplasmic domain (amino acids 1-18), a transmembrane region (amino acids 19-38), and an extracellular domain (amino acids 39-101).

The DNA of SEQ ID NO:3 lacks nucleotides 359 through 506 of the DNA of SEQ ID NO:1, and is thus designated the human TRAIL deletion variant (huTRAILdv) clone. The deletion causes a shift in the reading frame, which results in an in-frame stop codon after amino acid 101 of SEQ ID NO:4. The DNA of SEQ ID NO:3 thus encodes a truncated protein. Amino acids 1 through 90 of SEQ ID NO:2 are identical to amino acids 1 through 90 of SEQ ID NO:4. However, due to the deletion, the C-terminal portion of the huTRAILdv protein (amino acids 91 through 101 of SEQ ID NO:4) differs from the residues in the corresponding positions in SEQ ID NO:2.

The huTRAILdv protein lacks the above-described conserved regions found at the C-terminus of members of the TNF family of proteins. The inability of this huTRAILdv protein to cause apoptotic death of Jurkat cells further confirms the importance of these conserved regions for biological activity.

EXAMPLE 3: DNA encoding a murine TRAIL

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DNA encoding a murine TRAIL was isolated by the following procedure. A cDNA library comprising cDNA derived from the mouse T cell line 7B9 in the vector λZAP was prepared as described in Mosley et al. (*Cell* 59:335, 1989). DNA from the library was transferred onto nitrocellulose filters by conventional techniques.

5 Human TRAIL DNA probes were used to identify hybridizing mouse cDNAs on the filters. Two separate probes were used, in two rounds of screening. PCR reaction products about 400bp in length, isolated and amplified using the human TRAIL DNA as template, were employed as the probe in the first round of screening. These PCR products consisted of a fragment of the human TRAIL coding region. The

10 probe used in the second round of screening consisted of the entire coding region of the human TRAIL DNA of SEQ ID NO:1. A random primed DNA labeling kit (Stratagene, La Jolla, CA) was used to radiolabel the probes.

Hybridization was conducted at 37°C in 50% formamide, followed by washing with 1 x SSC, 0.1% SDS at 50°C. A mouse cDNA that was positive in both rounds of

15 screening was isolated.

The nucleotide sequence of this DNA is presented in SEQ ID NO:5 and the amino acid sequence encoded thereby is presented in SEQ ID NO:6. The encoded protein comprises an N-terminal cytoplasmic domain (amino acids 1-17), a transmembrane region (amino acids 18-38), and an extracellular domain (amino acids

20 39-291). This mouse TRAIL is 64% identical to the human TRAIL of SEQ ID NO:2, at the amino acid level. The coding region of the mouse TRAIL nucleotide sequence is 75% identical to the coding region of the human nucleotide sequence of SEQ ID NO:1.

EXAMPLE 4:   Antibodies that bind TRAIL

25 This example illustrates the preparation of monoclonal antibodies that specifically bind TRAIL. Suitable immunogens that may be employed in generating such antibodies include, but are not limited to, purified TRAIL protein or an immunogenic fragment thereof (e.g., the extracellular domain), fusion proteins containing TRAIL polypeptides (e.g., soluble TRAIL/Fc fusion proteins), and cells

30 expressing recombinant TRAIL on the cell surface.

Known techniques for producing monoclonal antibodies include those described in U.S. Patent 4,411,993. Briefly, mice are immunized with TRAIL as an immunogen emulsified in complete Freund's adjuvant, and injected in amounts ranging from 10-100 μg subcutaneously or intraperitoneally. Ten to twelve days later, the

35 immunized animals are boosted with additional TRAIL emulsified in incomplete Freund's adjuvant. Mice are periodically boosted thereafter on a weekly to bi-weekly immunization schedule. Serum samples are periodically taken by retro-orbital bleeding

or tail-tip excision for testing by dot blot assay or ELISA (Enzyme-Linked Immuno-sorbent Assay) for TRAIL antibodies.

Following detection of an appropriate antibody titer, positive animals are provided one last intravenous injection of TRAIL in saline. Three to four days later, the animals are sacrificed, spleen cells harvested, and spleen cells are fused to a murine myeloma cell line such as NS1 or, preferably, P3x63Ag 8.653 (ATCC CRL 1580). Fusions generate hybridoma cells, which are plated in multiple microtiter plates in a HAT (hypoxanthine, aminopterin and thymidine) selective medium to inhibit proliferation of non-fused cells, myeloma hybrids, and spleen cell hybrids.

The hybridoma cells are screened by ELISA for reactivity against purified TRAIL by adaptations of the techniques disclosed in Engvall et al. (*Immunochem.* 8:871, 1971) and in U.S. Patent 4,703,004. Positive hybridoma cells can be injected intraperitoneally into syngeneic BALB/c mice to produce ascites containing high concentrations of anti-TRAIL monoclonal antibodies. Alternatively, hybridoma cells can be grown *in vitro* in flasks or roller bottles by various techniques. Monoclonal antibodies produced in mouse ascites can be purified by ammonium sulfate precipitation, followed by gel exclusion chromatography. Alternatively, affinity chromatography based upon binding of antibody to protein A or protein G can be used, as can affinity chromatography based upon binding to TRAIL.

EXAMPLE 5:   DNA Laddering Apoptosis Assay

Human TRAIL was expressed and tested for the ability to induce apoptosis. Oligonucleotides were synthesized that corresponded to the 3' and 5' ends of the coding region of the human TRAIL gene, with SalI and NotI restriction sites incorporated at the ends of the oligonucleotides. The coding region of the human TRAIL gene was amplified by standard PCR techniques, using these oligonucleotides as primers. The PCR reaction products were digested with the restriction endonucleases SalI and NotI, then inserted into SalI/NotI-digested vector pDC409. pDC409 is an expression vector for use in mammalian cells, but is also replicable in *E. coli* cells.

pDC409 is derived from an expression vector designated pDC406 (described in McMahan et al., *EMBO J.* 10:2821, 1991, and in PCT application WO 91/18982, hereby incorporated by reference). pDC406 contains origins of replication derived from SV40, Epstein-Barr virus and pBR322 and is a derivative of HAV-EO described by Dower et al., *J. Immunol.* 142:4314 (1989). pDC406 differs from HAV-EO by the deletion of an intron present in the adenovirus 2 tripartite leader sequence in HAV-EO. DNA inserted into a multiple cloning site (containing a number of restriction

endonuclease cleavage sites) is transcribed and translated using regulatory elements derived from HIV and adenovirus. The vector also contains a gene that confers ampicillin resistance.

pDC409 differs from pDC406 in that a Bgl II site outside the mcs has been deleted so that the mcs Bgl II site is unique. Two Pme 1 sites and one Srf 1 site have been added to the mcs, and three stop codons (TAG) have been positioned downstream of the mcs to function in all three reading frames. A T7 primer/promoter has been added to aid in the DNA sequencing process.

The monkey kidney cell line CV-1/EBNA-1 (ATCC CRL 10478) was derived by transfection of the CV-1 cell line (ATCC CCL 70) with a gene encoding Epstein-Barr virus nuclear antigen-1 (EBNA-1) that constitutively expresses EBNA-1 driven from the human CMV intermediate-early enhancer/promoter, as described by McMahan et al., *supra*. The EBNA-1 gene allows for episomal replication of expression vectors, such as pDC409, that contain the EBV origin of replication.

CV1/EBNA cells grown in Falcon T175 flasks were transfected with 15 μg of either "empty" pDC409 or pDC409 containing the human TRAIL coding region. The transformed cells were cultured for three days at 37°C and 10% CO_2. The cells then were washed with PBS, incubated for 20 minutes at 37°C in 50 mM EDTA, scraped off of the flask with a cells scraper, and washed once in PBS. Next, the cells were fixed in 1% paraformaldehyde PBS for 10 minutes at 4°C, and washed 3x in PBS.

Jurkat cells were used as the target cells in this assay, to determine whether the TRAIL-expressing cells could induce apoptosis thereof. The Jurkat cell line, clone E6-1, is a human acute T cell leukemia cell line available from the American Type Culture Collection under accession no. ATCC TIB 152, and described in Weiss et al. (*J. Immunol.* 133:123-128, 1984). The Jurkat cells were cultured in RPMI media supplemented with 10% fetal bovine serum and 10 μg/ml streptomycin and penicillin to a density of 200,000 to 500,000 cells per ml. Four million of these cells per well were co-cultured in a 6 well plate with 2.5 mls of media with various combinations of fixed cells, supernatants from cells transfected with Fas ligand, and various antibodies, as indicated below.

After four hours the cells were washed once in PBS and pelleted at 1200 RPM for 5 minutes in a desktop centrifuge. The pellets were resuspended and incubated for ten minutes at 4°C in 500 μl of buffer consisting of 10 mM Tris-HCl, 10 mM EDTA, pH 7.5, and 0.2% Triton X-100, which lyses the cells but leaves the nuclei intact. The lysate was then spun at 4°C for ten minutes in a micro-centrifuge at 14,000 RPM. The supernatants were removed and extracted three times with 1 ml of 25:24:1 phenol-

chloroform-isoamyl alcohol, followed by precipitation with NaOAC and ethanol in the presence of 1 μg of glycogen carrier (Sigma).

The resulting pellets were resuspended in 10 mM Tris-HCl, 10 mM EDTA, pH 7.5, and incubated with 10 μg/ml RNase A at 37°C for 20 minutes. The DNA solutions were then resolved by 1.5% agarose gel electrophoresis in Tris-Borate EDTA buffer, stained with ethidium bromide and photographed while trans-illuminated with UV light.

The results were as follows. Fixed CV1/EBNA cells transfected with either pDC409 or pDC409-TRAIL produced no detectable DNA laddering. pDC409-TRAIL fixed cells co-cultured with Jurkat cells produced DNA laddering, but pDC409 fixed cells co-cultured with Jurkat cells did not.

DNA laddering was also seen when Jurkat cells were co-cultured with concentrated supernatants from COS cells transfected with DNA encoding human Fas ligand in pDC409. The supernatants are believed to contain soluble Fas ligand that is proteolytically released from the cell surface. The Fas ligand-induced DNA laddering could be blocked by adding 10 μg/ml of a soluble blocking monoclonal antibody directed against Fas. This same antibody could not inhibit laddering of Jurkat DNA by the pDC409-TRAIL cells, which indicates that TRAIL does not induce apoptosis through Fas.

In the same assay procedure, fixed CV1/EBNA cells transfected with pDC409-TRAIL induced DNA laddering in U937 cells. U937 (ATCC CRL 1593) is a human histiocytic lymphoma cell line. The ratio of effector to target cells was 1:4 (the same as in the assay on Jurkat target cells).

The fragmentation of cellular DNA into a pattern known as DNA laddering is a hallmark of apoptosis. In the foregoing assay, TRAIL induced apoptosis of a leukemia cell line and a lymphoma cell line.

EXAMPLE 6: Northern Blot Analysis

Expression of TRAIL in a number of different tissue types was analysed in a conventional northern blot procedure. Northern blots containing poly A^+ RNA from a variety of adult human tissues (multiple tissue northern blots I and II) were obtained from Clonetech (Palo Alto, CA). Other blots were prepared by resolving RNA samples on a 1.1% agarose-formaldehyde gel, blotting onto Hybond-N as recommended by the manufacturer (Amersham Corporation), and staining with methylene blue to monitor RNA concentrations. The blots were probed with an antisense RNA riboprobe corresponding to the entire coding region of human TRAIL.

Human TRAIL mRNA was detected in peripheral blood lymphocytes, colon, small intestine, ovary, prostate, thymus, spleen, placenta, lung, kidney, heart, pancreas, and skeletal muscle. TRAIL transcripts were found to be abundant in the large cell anaplastic lymphoma cell line Karpas 299 (Fischer et al., *Blood*, 72:234,

5   1988) and in tonsilar T cells. TRAIL message was present to a lesser degree in the Burkitt lymphoma cell line designated Raji.

TRAIL mRNA was not detected in testis, brain, or liver, or in several T cell lines. Little or no TRAIL transcripts were detected in freshly isolated peripheral blood T cells (PBT), either unstimulated or stimulated with PMA and calcium ionophore for

10   20 hours.

EXAMPLE 7:   Production of a Soluble TRAIL Polypeptide

A soluble human TRAIL polypeptide comprising amino acids 95 to 281 of SEQ ID NO:2 was prepared as follows. This polypeptide is a fragment of the

15   extracellular domain, lacking the spacer region discussed above.

An expression vector encoding soluble human TRAIL was constructed by fusing in-frame DNA encoding the following amino acid sequences (listed from N- to C-terminus): a leader sequence derived from human cytomegalovirus (CMV), a synthetic epitope designated Flag®, and amino acids 95-281 of human TRAIL. The

20   Flag® octapeptide (SEQ ID NO:7) facilitates purification of proteins fused thereto, as described above and in Hopp et al. (*Biotechnology* 6:1204-1210, 1988).

The TRAIL-encoding DNA fragment was isolated and amplified by polymerase chain reaction (PCR), using oligonucleotide primers that defined the termini of a DNA fragment encoding amino acids 95-281 of SEQ ID NO:2. The 3' primer was a 31-mer

25   that additionally added a NotI site downstream of the TRAIL-encoding sequence. The 5' primer added an SpeI site and a Flag® epitope encoding sequence upstream of the TRAIL-encoding sequence. PCR was conducted by conventional procedures, using the above-described human TRAIL cDNA as the template.

The reaction products were digested with SpeI and NotI, and inserted into the

30   expression vector pDC409 (described in example 5), which had been cleaved with SalI and NotI. Annealed oligonucleotides that form a SalI-SpeI fragment encoding a CMV open reading frame leader were also ligated into the vector. The amino acid sequence of the CMV-derived leader is presented as SEQ ID NO:9. Amino acids 1 to 29 of SEQ ID NO:9 are encoded by CMV DNA, whereas amino acids 30 to 32 are encoded by

35   oligonucleotides employed in constructing the vector. *E. coli* cells were transfected with the ligation mixture, and the desired recombinant expression vector was isolated therefrom.

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CV1-EBNA cells (ATCC CRL 10478; described in example 5) were transfected with the recombinant vector, which is designated pDC409-Flag-shTRAIL, and cultured to allow expression and secretion of the soluble Flag®-TRAIL polypeptide. Culture supernatants were harvested 3 days after transfection and applied to a column

5   containing an anti-Flag® antibody designated M2 immobilized on a solid support. The column then was washed with PBS. The monoclonal antibody M2 is described in Hopp et al., *supra*, and available from Kodak Scientific Imaging Systems, New Haven, Connecticut. 800µl fractions were eluted from the column with 50 mM citrate, and immediately neutralized in 0.45 ml 1M Tris (pH 8). Fractions were adjusted to

10   10% glycerol and stored at -20°C until needed.

This soluble recombinant Flag®/human TRAIL expressed in CV1/EBNA cells has an apparent molecular weight of 28 kD when analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The Flag® moiety contributes an estimated 880 daltons to the total molecular weight. Gel filtration analysis of purified soluble Flag®/TRAIL

15   suggests that the molecule is multimeric in solution with a size of ~80 kD. While not wishing to be bound by theory, the gel filtration analysis suggests that the soluble recombinant Flag®/human TRAIL naturally formed a combination of dimers and trimers, with trimers predominating.

An expression vector designated pDC409-Flag-smTRAIL, which encodes a

20   CMV leader-Flag®-soluble murine TRAIL protein, was constructed by analogous procedures. A DNA fragment encoding a soluble murine TRAIL polypeptide was isolated and amplified by PCR. Oligonucleotides that defined the termini of DNA encoding amino acids 99 to 291 of the murine TRAIL sequence of SEQ ID NO:6 were employed as the 5' and 3' primers in the PCR.

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EXAMPLE 8:   Lysis of Leukemia Cells by Soluble TRAIL

In example 5, cells expressing human TRAIL induced apoptosis of Jurkat cells (a leukemia cell line). In the following study, a soluble human TRAIL polypeptide killed Jurkat cells.

30   Jurkat cells were cultured to a density of 200,000 to 500,000 cells per ml in RPMI medium supplemented with 10% fetal bovine serum, 100 µg/ml streptomycin, and 100 µg/ml penicillin. The cells (in 96-well plates at 50,000 cells per well in a volume of 100 µl) were incubated for twenty hours with the reagents indicated in Figure 1. "TRAIL supe." refers to conditioned supernatant (10 µl per well) from

35   CV1/EBNA cells transfected with pDC409-Flag-shTRAIL (see example 7). "Control supe." refers to supernatant from CV1/EBNA cells transfected with empty vector. Where indicated, immobilized anti-Flag® antibody M2 ("Imm. M2") was added at a

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concentration of 10 µg/ml in a volume of 100 µl per well and allowed to adhere either overnight at 4°C or for 2 hours at 37°C, after which wells were aspirated and washed twice with PBS to remove unbound antibody. Jurkat cells treated with Fas ligand or M3, a blocking monoclonal antibody directed against Fas, (Alderson et al., *J. Exp. Med.* 181:71, 1995; and PCT application WO 95/10540) were included in the assay as indicated.

Metabolic activity of the thus-treated cells was assayed by metabolic conversion of alamar Blue dye, in the following procedure. Alamar Blue conversion was measured by adding 10 µl of alamar Blue dye (Biosource International, Camarillo, CA) per well, and subtracting the optical density (OD) at 550-600 nm at the time the dye was added from the OD 550-600 nm after four hours. No conversion of dye is plotted as 0 percent viability, and the level of dye conversion in the absence of TRAIL is plotted as 100 percent viability. Percent viability was calculated by multiplying the ratio of staining of experimental versus control cultures by 100.

The results are presented in Figure 1. Error bars represent the standard deviation of measurements from four independent wells, and the values are the average of these measurements.

The TRAIL-containing supernatant caused a significant reduction in viability of Jurkat cells. A greater reduction of cell viability resulted from contact with a combination of TRAIL-containing supernatant and immobilized anti-Flag® antibody M2. One possible explanation is that M2 facilitates cross-linking of the Flag®/TRAIL-receptor complexes, thereby increasing the strength of signaling.

Fas ligand demonstrated the ability to kill Jurkat cells. The anti-Fas antibody M3 inhibited the activity of Fas ligand, but not the activity of TRAIL.

In order to confirm that the changes in dye conversion in the alamar Blue assay were due to cell death, the decrease in cell viability induced by TRAIL was confirmed by staining the cells with trypan blue.

EXAMPLE 9: Lysis of Leukemia and Lymphoma Cells

In examples 5 and 8, TRAIL induced apoptosis of a leukemia cell line (Jurkat) and a lymphoma cell line (U937). The following study further demonstrates the ability of TRAIL to kill leukemia and lymphoma cells.

The human cell lines indicated in Table I were cultured to a density of 200,000 to 500,000 cells per ml in RPMI medium supplemented with 10% fetal bovine serum, 100 µg/ml streptomycin, and 100 µg/ml penicillin. The cells (in 96-well plates at 50,000 cells per well in a volume of 100 µl) were incubated for twenty hours with

conditioned supernatants (10 µl per well) from pDC409-Flag-shTRAIL transfected CV1/EBNA cells.

Metabolic activity was assayed by conversion of alamar Blue dye, in the assay procedure described in example 8. The results are presented in Table I.

5   In order to confirm that the changes in dye conversion in the alamar Blue assay were due to cell death, the decrease in cell viability induced by TRAIL was confirmed by staining the cells with trypan blue. Crystal violet staining, performed as described by Flick and Gifford (*J. Immunol. Methods* 68:167-175, 1984), also confirmed the results seen in the alamar Blue assay. The apoptotic nature of the cell death was

10   confirmed by trypan blue staining and visualization of apoptotic fragmentation by microscopy.

As shown in Table I, many cancer cell lines were sensitive to TRAIL mediated killing. The susceptibility of additional cell types to TRAIL mediated apoptosis can be determined using the assay procedures described in this examples section.

15   TRAIL exhibited no significant cytotoxic effect on the cell lines THP-1 , K562, Karpas 299, and MP-1. K299, also known as Karpas 299, (DSM-ACC31) was established from peripheral blood of a male diagnosed with high grade large cell anaplastic lymphoma (Fischer et al., *Blood*, 72:234, 1988). MP-1 is a spontaneously derived EBV-transformed B cell line (Goodwin et al., *Cell* 73:447, 1993). While not

20   wishing to be bound by theory, it is possible that these four cell lines do not express a receptor for TRAIL, or are characterized by upregulation of a gene that inhibits apoptosis.

Table 1. Effect of soluble TRAIL on cell line viability

Cell Line	Description	Percent Viability[a]
Bjab	Burkitt lymphoma	0.5 ± 3.8
Ramos	Burkitt lymphoma	12.1 ± 2.1
U937	histiocytic lymphoma	25.2 ± 8.2
HL60	promyelocytic leukemia	59.5 ± 3.2
Raji	Burkitt lymphoma	64.9 ± 4.5
Daudi	Burkitt lymphoma	70.2 ± 4.2
THP-1	monocytic cell line	92.3 ± 6.8
K562	chronic myelogenous leukemia	97.1 ± 4.8
K 299	large cell anaplastic lymphoma	99.0 ± 4.3
MP-1	spontaneous B cell line	104.9 ± 11.7

[a] Results are means ± SEMs of 4 wells for each data point

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EXAMPLE 10:   Cross-Species Activity of TRAIL

Interspecies cross-reactivity of human and murine TRAIL was tested as follows. Murine and human TRAIL were incubated with the human melanoma cell line A375 (ATCC CRL 1619). Since this is an adherent cell line, a crystal violet assay, rather than alamar Blue, was used to determine cell viability. A375 cells were cultured in DMEM supplemented with 10% fetal bovine serum, 100 μg/ml streptomycin, and 100 μg/ml penicillin. The cells (in 96-well plates at 10,000 cells per well in a volume of 100 μl) were incubated for 72 hours with the soluble murine TRAIL described in example 7. Crystal violet staining was performed as described by (Flick and Gifford (*J. Immunol. Methods* 68:167-175, 1984). The results demonstrated that both human and murine TRAIL are active on these human cells, in that murine and human TRAIL killed A375 cells.

The ability of human TRAIL to act on murine cells was tested, using the immortalized murine fibroblast cell line L929. Incubation of L929 cells with either human or murine TRAIL resulted in a decrease in crystal violet staining, thus demonstrating that human and murine TRAIL are active on (induced apoptosis of) murine cells. In addition to crystal violet, cell death was confirmed by trypan-blue staining.

EXAMPLE 11:   Lysis of CMV-Infected Cells

The following experiment demonstrates that the soluble Flag®-human TRAIL protein prepared in example 7 has a cytotoxic effect on virally infected cells.

Normal human gingival fibroblasts were grown to confluency on 24 well plates in 10% CO_2 and DMEM medium supplemented with 10% fetal bovine serum, 100 μg/ml streptomycin, and 100 μg/ml penicillin. Samples of the fibroblasts were treated as indicated in Figure 2. Concentrations of cytokines were 10 ng/ml for γ-interferon and 30 ng/ml of soluble Flag®-human TRAIL. All samples receiving TRAIL also received a two-fold excess by weight of anti-Flag® antibody M2 (described above), which enhances TRAIL activity (presumably by crosslinking).

Pretreatment of cells with the indicated cytokines was for 20 hours. To infect cells with cytomegalovirus (CMV), culture media were aspirated and the cells were infected with CMV in DMEM with an approximate MOI (multiplicity of infection) of 5. After two hours the virus containing media was replaced with DMEM and cytokines added as indicated. After 24 hours the cells were stained with crystal violet dye as described (Flick and Gifford, 1984, *supra*). Stained cells were washed twice with water, disrupted in 200 μl of 2% sodium deoxycholate, diluted 5 fold in water, and the OD taken at 570 nm. Percent maximal staining was calculated by normalizing ODs to

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the sample that showed the greatest staining. Similar results were obtained from
several independent experiments.

The results presented in Figure 2 demonstrate that TRAIL specifically killed
CMV infected fibroblasts. This cell death was enhanced by pretreatment of the cells

5   with γ-interferon. No significant death of non-virally infected fibroblasts resulted from
contact with TRAIL.